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Proteomic analysis of dorsolateral prefrontal cortex indicates the involvement of cytoskeleton, oligodendrocyte, energy metabolism and new potential markers in schizophrenia

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ABSTRACT

Schizophrenia is likely to be a consequence of serial alterations in a number of genes that, together with environmental factors, will lead to the establishment of the illness. The dorsolateral prefrontal cortex (Brodmann's Area 46) is implicated in schizophrenia and executes high functions such as working memory, differentiation of conflicting thoughts, determination of right and wrong concepts, correct social behavior and personality expression. We performed a comparative proteome analysis using two-dimensional gel electrophoresis of pools from 9 schizophrenia and 7 healthy control patients' dorsolateral prefrontal cortex aiming to identify, by mass spectrometry, alterations in protein expression that could be related to the disease. In schizophrenia-derived samples, our analysis revealed 10 downregulated and 14 upregulated proteins. These included alterations previously implicated in schizophrenia, such as oligodendrocyte-related proteins (myelin basic protein and transferrin), as well as malate dehydrogenase, aconitase, ATP synthase subunits and cytoskeleton-related proteins. Also, six new putative disease markers were identified, including energy metabolism, cytoskeleton and cell signaling proteins. Our data not only reinforces the involvement of proteins previously implicated in schizophrenia, but also suggests new markers, providing further information to foster the comprehension of this important disease.

1. Introduction

Previous studies of global gene expression in different brain regions of schizophrenia (SCZ) patients revealed dysfunctions in synaptogenesis and neural plasticity (Mirnics et al., 2000; Hakak et al., 2001; Vawter et al., 2001; Aston et al., 2004; Arion et al., 2007; Smalla et al., 2008), energy metabolism (Vawter et al., 2001; Middleton et al., 2002; Prabakaran et al., 2004), cytoskeleton assembly (Hakak et al., 2001; Vawter et al., 2001; Tkachev et al., 2003) and oligodendrocyte metabolism (Tkachev et al., 2003; Aston et al., 2004; Katsel et al., 2005; Arion et al., 2007). Alterations on these pathways have been confirmed by some proteomic studies, performed in brain regions such as the anterior cingulate cortex (Clark et al., 2006), the prefrontal cortex (Novikova et al., 2006) and the corpus callosum (Sivagnanasundaram et al., 2007). Proteomic studies also pointed to alterations in synaptic functions in SCZ, in two recent publications (Pennington et al., 2007; Behan et al., 2008).

Prefrontal cortex (PFC) is most elaborated in primates, where it provides a diverse and flexible behavioral repertoire, including the so called "executive functions" (Miller and Cohen, 2001). Executive functions include differentiation of conflicting thoughts, determination of concepts of good and bad, perspectives in accordance with determined actions, moderating correct social behavior, future consequences of current activities as well as working memory. Basically, the activities of this region involve the organization of thoughts and actions according to internal aims (cognitive control) (Miller and Cohen, 2001; Liston et al., 2006). Dysfunction of the dorsolateral PFC (DLPFC) has been implicated in the

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pathophysiology of SCZ by many groups (Weinberger, 1996; Barch, 2005; Barbas and Zikopoulos, 2007).

We performed comparative proteomic analysis of the left DLPFC (Brodmann's Area 46) tissue of a pool of nine SCZ patients, which was contrasted to a pool of seven paired controls, using two-dimensional gel electrophoresis (2-DE), followed by MALDI-TOF/TOF mass spectrometry. The regulated proteins found here included proteins that were previously reported as abnormal in SCZ, as well as other new potential protein markers that may be involved in the pathobiology of the disease.

2. Materials and methods

2.1. Human dorsolateral prefrontal cortex samples (DLPFC)

Post-mortem brain samples from the left DLPFC tissue (BA46) were collected from nine schizophrenia patients and seven controls, which were free from psychiatry disorders, somatic diseases or brain tumors and were never treated with antidepressant or antipsychotic medications. Brain samples were dissected by an experienced neuropathologist (on average 24.3 h after death) and deep-frozen immediately after collection.

All samples were obtained from the brain bank of the Central Institute of Mental Health (Mannheim, Germany). Controls were collected at the Institute of Neuropathology, Heidelberg University, and their clinical records were collected from their relatives and general practitioners. Patient samples derived from in-patients of the Mental State Hospital Wiesloch, Germany. All cases and controls were German whites. All SCZ patients have been long-term in-patients at the Mental State Hospital Wiesloch, Germany, and the diagnosis of schizophrenia was made *ante mortem* by an experienced psychiatrist according to the DSM IV criteria (American, 1994). For each patient the antipsychotic treatment history was assessed by examining the medical charts and calculated in chlorpromazine equivalents (CPE), through the algorithm developed by Jahn and Mussgay (1989). All patients and controls underwent neuropathologic characterization to rule out associated neurovascular or neurodegenerative disorders. The classification according to Braak was stage II or less for all subjects (Braak and Braak, 1991; Braak et al., 2006). Patients and controls had no history of alcohol, drug abuse, or severe physical illness. All assessment and *post-mortem* evaluations and procedures were previously approved by the ethics committee of the Faculty of Medicine of Heidelberg University, Germany. Detailed patient information is given in Table 1.

2.2. Sample preparation

Fifty milligrams of human DLPFC (gray matter) were individually homogenized in 1.5 ml tubes with glass spheres in 200 μl of 6 M Guanidine·HCl and 0.1 M HEPES buffer. Samples were centrifuged for 10 min at 14,000 rpm and quantified (Bradford, 1976) to prepare equimolar pools. To achieve a similar final quantity of protein (650 μg), control pools were made with 92.9 μg of protein from each of the seven samples, whereas SCZ pools were made with 72.2 μg of protein from each of the nine samples.

2.3. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis were carried out as described by Martins et al. (2007) applying 650 μg of pooled proteins from SCZ or control samples to IPG gel strips with a nonlinear separation range of pH 3–10, 4–7 and 6–11.

Proteins were detected by a colloidal coomassie staining protocol (Candiano et al., 2004) due to its superior reproducibility and sensitivity when compared with other protein colorimetric post-electrophoresis staining methods in visible light (Westermeier and Marouga, 2005). All experiments were performed in triplicate. Only proteins that appeared to be differentially expressed in all triplicates were considered as differentially regulated.

2.4. Image analysis – determination of quantitative differences

ImageMaster 2 D software, version 3.01 (GE Healthcare) was used for spot detection and pI/MW calibration of DLPFC 2-DE gels by comparison with known 2D and 1D marker proteins (Bio-Rad, Hercules, CA). MW and pI values of the markers were determined using first-order LaGrange regression. Moreover, the spots volumes from SCZ and CTRL 2-DE profiles were determined and the correspondent spots were matched. Protein spots with a mean *n*-fold change between SCZ and control DLPFC gels of +/− 1.3 were excised for identification by mass spectrometry (MS).

2.5. Protein identification by peptide mass fingerprinting

Differentially expressed proteins were excised and subjected to MS-based identification. Peptides were generated and extracted from the gel-separated proteins following established in-gel trypsin digestion protocols (Shevchenko et al., 1996).

Mass spectra from each spot were acquired using an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with N_2 laser (λ: 337 nm) in positive reflector mode and a 20 kV acceleration voltage. One hundred and 1000 shots were accumulated, respectively, for MS and MS/MS spectra. Flexcontrol version 2.2 software (Bruker Daltonics) was used to acquire and process MS and MS/MS data.

Acquired MS/MS spectra were searched against the NCBI database (Dec 16th, 2006) using an in-house version of MASCOT search engine 2.1 (Matrix Sciences, London, UK). Parameters used were as follows: trypsin as enzyme allowing one missed cleavage, carbamidomethylation and oxidized methionine as fixed modifications. The mass accuracies of the precursor and fragment ions were 150 ppm and 0.7 Da, respectively.

2.6. Western blot

One hundred microgram of total protein from DLPFC of each individual patient were run on a 12.5% SDS minigel (Bio-Rad, Hercules, CA, USA) and transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA) at 100 V for 1 h with cooling system. The membrane was treated with 5% Carnation instant nonfat dry milk in TBS-T for 4 h and rinsed in TBS-T 3 times for 20 min. The membrane was then incubated with anti-GFAP (Abcam, Cambridge, UK) at a 1:500 dilution in TBS-T overnight at 4 °C. The membrane was washed with water and TBS-T for 15 min. Incubations with anti-c-MYC-peroxidase antibody (GE Healthcare, Uppsala, Sweden) were carried out for 40 min at room temperature after which the membrane was washed with water and TBS-T. Finally the membrane was incubated with ECL mixture (GE Healthcare, Uppsala, Sweden) for 1 min and exposed to ECL film (GE Healthcare, Uppsala, Sweden). The membrane was scanned, and the band signal (optical density) was assessed using QuantityOne software (Bio-Rad, Hercules, CA, USA). The significant changes of individual samples between SCZ patients and controls were determined using the Student's two-tailed *t*-test.

3. Results

2-DE profiles of pooled samples were generated in triplicate. On average, 750 spots were detected in each 2-DE (Fig. 1). When SCZ

Table 1
Patient and control clinical data. Abbreviations: atyptyp: duration of atypical treatment/duration of treatment with typical neuroleptics during lifetime; CPE: medication calculated in chlorpromazine equivalents (mg); CPE last ten years: the sum of medications during the last ten years in kg; Hosp: Hospitalization time in years; ECT: electroconvulsive therapy. Using Mann–Whitney test, we found no significant differences between patients and controls for age ($p = 0.1385$), PMI ($p = 0.4587$) and pH ($p = 0.6338$). Using exact Fisher test, no significant differences were found between the patients and controls gender ($p = 0.3575$).

Sample ID	Case	Age (years)	Gender	PMI (h)	pH values	Type of SCZ	Duration of disease (years)	Duration of medication (years)	atyptyp	CPE last dosis	CPE last ten years	Cause of death	DSM IV	Age at onset	Last medication	Cigarettes	Alcohol	Hosp	ECT
13/00	SCZ	64	F	11	6.7	Residual, chronic paranoid episodes	48	45	3	1536	7.7	Pulmonary insufficiency	295.6	16	Clozapine 500 mg, haloperidol 40 mg, ciatyl 40 mg	0	No	21	Yes
36/02	SCZ	73	M	20	6.6	Residual, chronic paranoid episodes	43	40	1	507.4	1.7	Heart infarction	295.6	30	Perphenazine 32 mg, promethazine 150 mg	30/day	No	33	No
39/02	SCZ	43	M	18	6.9	Residual, chronic paranoid episodes	22	20	2	464	2.6	Heart infarction	295.6	20	Zuclopethixol 40 mg, valproate 1200 mg, tiapride 300 mg	0	No	13	No
39/03	SCZ	77	F	32	6.5	Residual, chronic paranoid episodes	49	48	2	2555	8.3	Lung embolism	295.6	28	Clozapine 400 mg, benperidol 25 mg, chlorprothixen 150 mg	0	No	48	Yes
43/03	SCZ	76	F	17	6.8	Residual, chronic paranoid episodes	49	47	1	300	4.9	Cardio-pulmonary insufficiency	295.6	27	Perazine 300 mg	0	No	30	Yes
46/00	SCZ	63	F	31	6.8	Residual, chronic paranoid episodes	40	30	3	75	1.8	Heart infarction	295.6	24	Olanzapine 15 mg	30/day	No	30	Yes
50/01	SCZ	81	M	4	7.1	Residual, chronic paranoid episodes	62	50	1	92.8	1.4	Cor pulmonale, heart insufficiency	295.6	19	Haloperidol 4 mg, prothypendyl 80 mg	20/day	No	48	No
75/02	SCZ	92	F	37	6.9	Residual, chronic paranoid episodes	51	48	1	100	3.4	Pancreas-carcinoma	295.6	41	Prothipendyl 160 mg, perazine 100 mg	0	No	51	No
83/01	SCZ	71	M	28	6.4	Residual, chronic paranoid episodes	40	35	1	782.4	10	Heart infarction	295.6	30	Haloperidol 32 mg, pipamperone 40 mg	40/day	No	12	No
02/02	Control	41	M	7	6.5							Heart infarction				0	No		
43/01	Control	91	F	16	6.7							Cardio-pulmonary insufficiency				0	No		
50/02	Control	69	F	96	6.4							Lung embolism				0	No		
51/02	Control	57	M	24	6.9							Heart infarction				0	No		
57/02	Control	53	M	18	7.0							Heart infarction				0	No		
59/02	Control	63	M	13	6.5							Heart infarction				0	No		
61/01	Control	66	M	16	6.8							Heart infarction				0	No		

and control pools were compared, 22 spots (~3%) were consistently identified with significant changes in relative abundance (>1.3 fold difference) (Table 2). These 22 spots revealed 24 distinct proteins with apparent altered regulation, being 10 downregulated and 14 upregulated in SCZ samples (Fig. 1). Sixteen proteins were identified as single spots, four proteins were identified in multiple spots (spots 8, 11, 19, 20 – Fig. 1) and four proteins were identified in two spots (the spots 9 and 16 were doublets). All 24 proteins were successfully identified by MALDI-TOF/TOF and grouped according to their functional classes using the Human Protein Reference Database (HPRD – http://www.hprd.org) (Table 2).

4. Discussion

4.1. Differentially expressed proteins

The present findings support previous reports on abnormalities of some proteins in SCZ, and suggest further new targets which may be relevant for the pathobiology of the disease.

4.1.1. Oligodendrocyte metabolism

We found in SCZ-DLPFC the differential expression of two oligodendrocyte-related proteins: myelin basic protein (MBP: downregulated: 1.5x) and transferrin (TF: downregulated: 2.7x). MBP, a major constituent of the myelin sheath in the CNS, is controlled by Brain-derived neurotrophic factor (BDNF) (Hohlfeld et al., 2000) and has a neuroprotective role in vivo (Moalem et al., 1999). TF is an iron carrier that participates in oligodendroglial cell differentiation, maturation and function (Espinosa de los Monteros et al., 1999; Paez et al., 2005). Both proteins were previously shown to be associated with SCZ by other groups (Table 3).

The main function of oligodendrocytes is the maintenance of axon myelinization in the central nervous system (CNS). The diminution or malformation of the myelin sheath in CNS can lead to a reduced propagation of nerve impulses, reducing the neuronal connectivity (Davis et al., 2003) and triggering an immune response that can compromise tissue functioning (Kuritzky et al., 1976). The identification of these two myelin-related proteins supports previously reported alterations of function, distribution and density of oligodendrocytes in SCZ. Approaches as diverse as diffusion tensor (Buchsbaum et al., 1998), magnetic transfer (Foong et al., 2000) or magnetic resonance imaging (Cannon et al., 1998; Sallet et al., 2003) allowed the observation of white matter abnormalities in SCZ, that were reinforced by gene expression (Hakak et al., 2001; Vawter et al., 2001; Tkachev et al., 2003; Prabakaran et al., 2004; Arion et al., 2007) and proteomics (Prabakaran et al., 2004), all suggesting oligodendrocyte-related alterations. While many of these alterations could be due to a reduced transcriptional activity of oligodendrocyte-related genes, there is accumulating evidence that this effect may be primarily due to a loss of oligodendrocytes or to an abnormal coherence/organization of fiber tracts that would lead to myelin-axonal disruption in SCZ brains (Hof et al., 2003; Kubicki et al., 2005). The reduced expressions of MBP and TF in our SCZ samples further suggest that an abnormality of connectivity may be related to the biology of the disease (Davis et al., 2003).

4.1.2. Dysregulation of energy metabolism

We found the differential regulation of proteins that participate in glucose metabolism through glycolysis (aldolase C - ALDOC; upregulated 1.8x and phosphoglycerate kinase 1 - PGK1; upregulated 1.4x) and Krebs cycle (aconitase 2 precursor - ACO2; upregulated 1.9x), beyond cytosolic malate dehydrogenase (MDH1 - downregulated 1.3x) that has important roles in the citrate shuttle/malate–aspartate shuttle, one of the systems which links cytosol and mitochondria metabolisms. The downregulation of MDH1 in SCZ, previously observed (Vawter et al., 2004a; Middleton et al., 2002), may lead to an increasing in cytosolic H+, modifying activity of 6-phosphofructokinase, decreasing the energy production pathways via glycolysis (Vawter et al., 2004a).

We detected the differential regulation of two ATP synthase subunits (ATP5H; downregulated 1.4x and ATP5A1 – identified by two spots - upregulated: 2x and 1.4x) and ubiquinol-cyto-

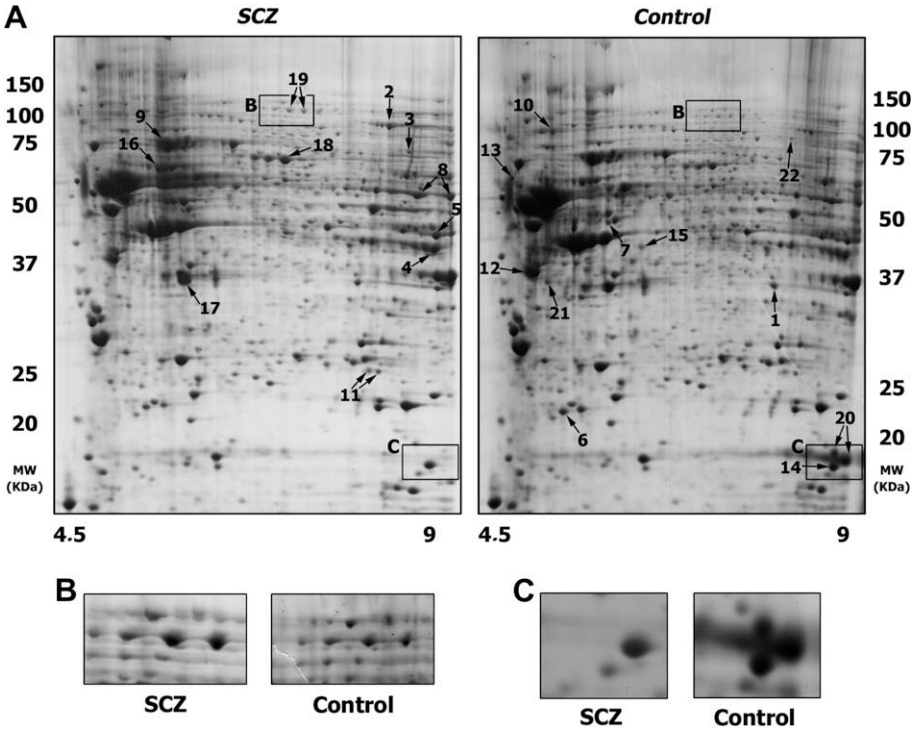

Fig. 1. A-) 2-DE profile of SCZ pooled samples and control pooled samples. The upregulated proteins are indicated by arrows. 1B and 1C represent enlarged sections of the 2-DE.

Table 2
Proteins regulated in schizophrenia samples, classified according to their biological function.

Biological process	Reg. in SCZ	Fold change	Spot	Accession	Protein name	pI (th)	MW (th)	Chr. locus	Pept. matched	MASCOT score
Metabolism/energy pathways	↓	−1.33	1	gi|5174539	Cytosolic malate dehydrogenase; soluble malate dehydrogenase	6.91	36426	2p13.3	12	370
	↑	1.88	2	gi|4501867*	Aconitase 2 precursor; aconitate hydratase; citrate hydro-lyase	7.36#	85425	22q13.2-q13.31	29	335
	↑	4.44	3	gi|388891	Transketolase	7.89#	67877	3p14.3	28	102
	↑	1.82	4	gi|30582851	Aldolase C, fructose-bisphosphate	6.41	39456	17cen-q12	25	440
	↑	1.42	5	gi|4505763	Phosphoglycerate kinase 1	8.30#	44615	Xq13	47	616
	↓	−1.43	6	gi:5453559	(ATP5H) ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d isoform a	5.21+	18491	17q25	13	213
	↓	−1.64	7	gi:92090651	Ubiquinol-cytochrome-c reductase complex core protein I, mitochondrial precursor	5.94+	53270	3p21.3	17	236
	↑	−2.00 and −1.48	8	gi:50345980	(ATP5A1) ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit isoform b	8.24	54574	18q12-q21	26	346
	↑	1.65	9	gi|15341906	(ATP6V1A) ATPase, H+ transporting, lysosomal 70 kD, V1 subunit a, isoform 1	5.35+	68660	3q13.2-q13.31	23	251
Protein metabolism	↓	−2.29	10	gi:83699649	Heat shock 90 KDa protein 1, alpha	5.07	98113	14q32.33	20	233
	↑	1.65	9	gi|5729877*	Heat shock 70 KDa protein 8 isoform 1; heat shock cognate protein, 71 KDa; heat shock 70 KDa protein 1	5.37	71082	11q24.1	34	211
	↑	2.57 and 2.30	11	gi:14250587*	Protein-L-isoaspartate (D-aspartate) O-methyltransferase	6.7	24806	6q24-q25	9	245
	↑	2.05	16	gi:31542947	Chaperonin; mitochondrial matrix protein P1; P60 lymphocyte protein; heat shock 60 kD protein 1	5.7	61187	2q33.1	25	338
Cell growth/ maintenance	↑	1.68	12	gi|38566198	Glial fibrillary acidic protein (GFAP)	5.42+	49776	17q21	44	395
	↓	−2.10	13	gi|24658018*	Neurofilament, light polypeptide (NEFL)	4.64+	61536	8p21	20	80
	↓	−5.30	14	gi:30582531*	Cofilin 1 (nonmuscle)	8.22#	18719	11q13	14	172
	↓	−1.55	15	gi:4501887	Actin, gamma 1 propeptide; actin, cytoplasmic 2	5.31	42108	17q25	21	354
	↑	2.05	16	gi|14249342	Internexin neuronal intermediate filament protein, alpha; neurofilament-66	5.34	55528	10q24.33	22	140
Cell communication/ signal transduction	↑	1.43	17	gi|30583449	Guanine nucleotide binding protein (G protein), beta polypeptide 1	5.6+	38151	1p36.33	14	246
	↑	1.50	18	gi:4503377*	Dihydropyrimidinase-like 2; collapsin response mediator protein hCRMP-2	5.95+	62711	8p22-p21	33	514
	↑	2.50 and 2.14	19	gi|39795292	Dynamin 1	6.57	96379	9q34	35	318
Oligodendrocyte metabolism	↓	Absent and −1.52	20	gi|37590005	Myelin basic protein	11.11#	19453	18q23	22	323
	↓	−2.70	22	gi:37747855	Transferrin	6.97	79310	3q22.1	35	288
Osmoregulation/ hormone metabolism	↓	−1.77	21	gi|4503065	Crystallin, mu; NADP-regulated thyroid-hormone binding protein	5.06+	33925	16p13.11-p12.3	8	137

Proteins marked with (*) are encoded by genes that map to genomic regions previously associated with schizophrenia. Proteins presented in this table were identified in 3–10NL profiles; proteins marked with (#) were also found in 6–11 maps and proteins marked with (+) were also found in 4–7 maps. The accession numbers are from the NCBI database.

chrome-c reductase complex core protein I (UQCRC1; downregulated 1.6x - not previously described in SCZ) suggesting a lack in oxidative phosphorylation, which can contribute to an oxidative damage.

The oxidative stress can lead to DNA damage, protein inactivation, altered gene expression and apoptotic events. These processes start during the neurodevelopment, and their close connection to neuronal plasticity strongly suggest that an energetic component may be relevant to the pathogenesis of SCZ (Ben-Shachar, 2002; Ben-Shachar and Laifenfeld, 2004; Mahadik et al., 2006). The differential expression of genes and proteins related to the energy metabolism has been previously described by large-scale studies in brains of SCZ patients, (cited in introduction) and is supported by our findings. Studying the synaptic proteome of SCZ, Smalla et al. (2008) also found the differential regulation of ALDOC and ATP5H.

There is no consensus whether these alterations are causative or consequences of a pathological process. Despite the energy metabolism alterations found in SCZ could be an effect of medication (Beasley et al., 2006), alterations in the glucose metabolism have been extensively reported as a central component of the disease and not a simple antipsychotic effect (Stone et al., 2004). Moreover, a correlation between the differential expression of glucose metabolism enzymes and oxidative phosphorylation enzymes has been recently described (Ben-Shachar et al., 2007).

4.1.3. Defects in protein metabolism and dysregulation of immune system

Heat shock proteins (HSP) play numerous roles in cellular metabolism. Whereas an abnormal immune response against heat shock 90 KDa protein 1 alpha (HSPCA; downregulated 2.3x) was described in SCZ (Kim et al., 2001), some SCZ patients also present antibodies reactive to heat shock 60 kD protein 1 (HSPD1; upregulated 2x), further implicating these proteins in the SCZ pathobiology.

Table 3
Proteins found regulated in DLPFC SCZ previously described by other SCZ tissues analysis.

Gene symbol	Product name	Type of analysis	Tissue	Described as regulated by:
MBP*	Myelin basic protein	1-Immunoassay 2-Microarray and qPCR	1-Anterior frontal cortex 2-Prefrontal cortex	1-Honer et al., 1999 2-Tkachev et al., 2003
TF*	Transferrin	1-Microarray 2-Microarray 3-Microarray and proteomics 4-Microarray 5-In situ hybridization	1-Prefrontal cortex 2-Prefrontal cortex 3-Prefrontal cortex 4-Prefrontal cortex 5-Anterior cingulate cortex	1-Hakak et al., 2001 2-Tkachev et al., 2003 3-Prabakaran et al., 2004 4-Arion et al., 2007 5-McCullumsmith et al., 2007
ALDOC*	Fructose bisphosphate aldolase C	1-Immunoassay* 2-Proteomics 3-Microarray and proteomics 4-Proteomics	1-Cerebrospinal fluid 2-Frontal cortex 3-Prefrontal cortex 4-Anterior cingulate cortex	1-Willson et al., 1980 2-Johnston-Wilson et al., 2000 3-Prabakaran et al., 2004 4-Clark et al., 2006
ATP5A1*	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit isoform b	qPCR	Hippocampus	Altar et al., 2005
GFAP*	Glial fibrillary acidic protein	1-Proteomics 2-In situ hybridization 3-Microarray and qPCR	1-Frontal cortex 2-Cingulate cortex 3-Prefrontal cortex	1-Johnston-Wilson et al., 2000 2-Webster et al., 2005 3-Tkachev et al., 2003
MDH	Malate dehydrogenase	1-Microarray 2-Microarray 3-Microarray	1-Prefrontal cortex 2-Prefrontal cortex 3-Lymphocytes	1-Middleton et al., 2002 2-Vawter et al., 2004a 3-Vawter et al., 2004b -2
ATP6V1A	ATPase, H+ transporting, lysosomal 70 kD, V1 subunit A, isoform 1	cDNA array	Hippocampus	Altar et al., 2005
CRYM	Crystallin	1-Microarray 2-Microarray 3-Proteomics	1-Prefrontal Cortex 2- prefrontal cortex 3-Corpus callosum	1-Hakak et al., 2001 2-Arion et al., 2007 3-Sivagnanasundaram et al., 2007
NEFL	Neurofilament triplet L protein	Proteomics	Corpus callosum	Sivagnanasundaram et al., 2007
DNM1	Dynamin	Proteomics	Anterior cingulate cortex	Clark et al., 2006
DPYSL2	Dihydropyrimidinase-like 2	Proteomics	Frontal cortex	Johnston-Wilson et al., 2000

Genes/proteins marked with (*) were validated by corresponding authors.

Transgenic mice of protein-L-isoaspartate (D-aspartate) O-methyltransferase (PCMT1 - identified by two spots - upregulated: 2.6x and 2.3x) have abnormal cortical activities and seizures that could not be controlled by antiepileptic drugs (Kim et al., 1999). These animals also helped to reveal synapsin I as the major substrate of this methyltransferase. These findings not only associate PCMT1 to SNC development, but also implicate this protein in the synapses function, two aspects strongly altered in SCZ. Other methyltransferases were previously implicated in SCZ pathogenesis, such as COMT, that plays a role dopamine release in PFC (Gogos and Gerber, 2006).

4.1.4. Cytoskeleton-related abnormalities

One-quarter of the differentially expressed proteins identified here are cytoskeleton proteins, which altered expression can directly intervene in cellular aspects such as shape, and neurotransmission, which are essential for neurons physiology (reviewed in Benitez-King et al., 2004).

Internexin (INA; upregulated 2x), firstly reported in SCZ, is an important player in neuronal intermediate filament inclusion disease (Cairns et al., 2004). INA interacts with glial fibrillary acidic protein (GFAP; upregulated 1.7x), which was demonstrated to be altered in astrocytes of SCZ, bipolar disorder, and depression (Miguel-Hidalgo et al., 2000; Webster et al., 2001; Pennington et al., 2007). INA also interacts with the neurofilament light polypeptide (NEFL; downregulated 2.1x, in agreement with the findings of Pennington et al., 2007) that is directly involved in NMDA receptor function (Ehlers et al., 1995), in conformity with previously described glutamatergic SCZ dysfunction (Meador-Woodruff and Healy, 2000; Olney and Farber, 1995). Moreover, our findings are in line with the proposed rat model to SCZ (ketamine-treated) that confirms the hypofunction of NMDA receptor-mediated signaling as well as the differential regulation of NEFL and Internexin (Smalla et al., 2008) as we found. The glutamatergic dysfunction can modulate dynamin (DNM1 – identified by two spots; upregulated: 2.5x and 2.1x) levels, that was also described as differentially expressed in DLPFC-BA9 (Pennington et al., 2007). Beyond the interaction with INA, GFAP altered expression can compromise the key role of astroglia in signaling and transmission (Haydon, 2001). The upregulation of GFAP in SCZ samples, was confirmed by western blot in individual samples ($p = 0.015$), and reinforces its potential role in SCZ (Fig. 2).

Cofilin 1 (CFL1 – spot 14 – downregulated 5.3x), not previously related to SCZ, is an actin filament-related protein that interacts with BDNF, a myelin basic protein expression modulator (Hohlfeld et al., 2000) described as related to SCZ by many groups.

Crystallin (CRYM – spot 21 – downregulated 1.8x) interacts with GFAP in the brain (Nicholl and Quinlan, 1994) and modulates cytoskeleton assembly (Liang and MacRae, 1997). CRYM protein was found regulated in astrocytes associated with senile plaques and cerebral amyloid angiopathy in AD patients (Wilhelmus et al., 2006) and in gene expression and proteome analysis (Hakak et al., 2001; Arion et al., 2007; Sivagnanasundaram et al., 2007). Other cytoskeleton-related proteins previously described to be altered in SCZ (Neurofilament light polypeptide and actin gamma 1 propeptide) were also found in our SCZ-DLPFC samples (Table 2).

4.1.5. Cellular signaling

Evidence for G-protein involvement in SCZ has been reported (Catapano and Manji, 2006). Guanine nucleotide binding protein - beta polypeptide 1 (GNB1; upregulated 1.4x) is a G protein subunit, and dihydropyrimidinase-like 2 (DPYSL2; upregulated 1.5x) is an important player in neuronal development, which may influence SCZ susceptibility (Hong et al., 2005).

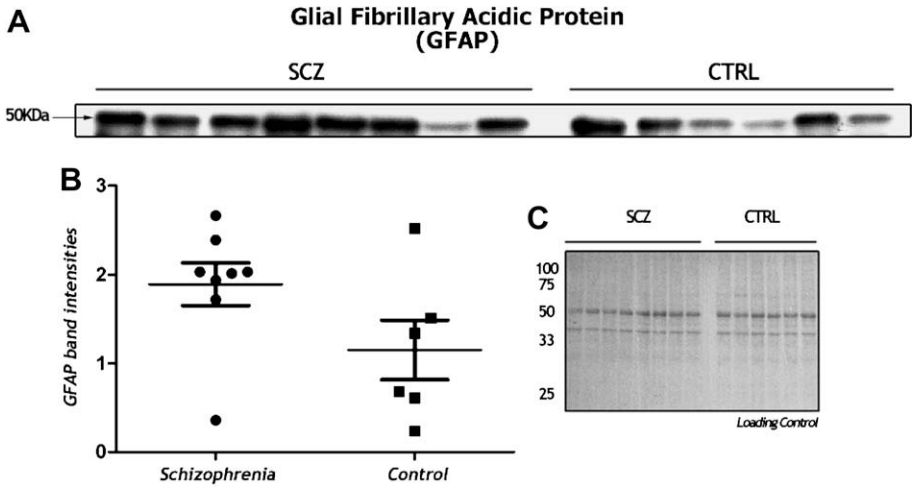

Fig. 2. The validation of the potential SCZ biomarker Glial Fibrillary Acidic Protein (GFAP) using western blot (WB). A) GFAP WB profile in 8 SCZ and 6 CTRL samples; B) The densitometric data from GFAP bands. C) Ponceau-stained membrane as a loading control.

4.2. Analysis of protein pools

A number of reasons prompted us to compare protein pools instead of individual protein extracts. While we are aware that more dramatic alterations in certain proteins of a single individual might 'contaminate' the pool, suggesting unreal alterations, we believe that the advantages of sample pooling may overcome its disadvantages. The first clear advantage of sample pooling is the possibility of reducing individual proteome variations (not related to the disease), while highlighting the most consistent (disease-related) alterations. Other advantages include an important reduction in the amount of protein required from each sample, allowing experimental replicates and subsequent studies. This approach has been successfully used by different authors not only for proteomics (Jiang et al., 2003; Lehmensiek et al., 2007a,b), but also for gene expression analysis (Vawter et al., 2001; Katsel et al., 2005).

4.3. Confounding factors

We would like to emphasize that, as all the findings described here derived from a relatively small group of samples, and no replications were done with an independent sample set, this limitation of our study should be taken into account. Nevertheless, we could validate in individual samples the differential expression of GFAP, suggesting it as a potential SCZ biomarker. Moreover, we should be careful to affirm that all the protein alterations detected in our analyses are directly associated with SCZ. If associated with SCZ, we should also be careful in announcing that their regulation is causative of the disease process or a consequence of the disease. It is important to be aware that some confounding factors such as age, gender, diet or the medications used by the patients may reflect in the proteome alterations observed here.

We should note that all the SCZ samples used here came from patients that were using antipsychotics. This limitation of the present and many other studies certainly has important effects on proteome alterations. As shown in Table 1, three out of nine patients from our study were using haloperidol at death. Sugai et al. (2004), using cDNA arrays from cynomolgus monkeys and Narayan et al. (2007), using in situ hybridization analysis of mice, showed that myelin basic protein (MBP) is modulated by haloperidol. An altered expression of apolipoprotein A-I in plasma of SCZ medicated patients was also found (La et al., 2007), whereas malate dehydrogenase, peroxiredoxin 3, vacuolar ATP synthase subunit beta and mitogen-activated protein kinase kinase 1 were found regulated in hippocampus of chlorpromazine/clozapine treated rats (La et al., 2006). However, a considerable percentage of the proteins identified here have not been reported to be associated with any of the drugs used by the patients studied and many have been associated with SCZ processes largely independent of an exogenous drug effect, such as genetic linkage studies. We expected that the pooling strategy adopted here could contribute to dilute the protein alterations driven by haloperidol in 1/3 of our patients, but we cannot exclude that some of the alterations seen here could be drug-related, rather that SCZ-related.

Whereas the study of brain samples derived from psychotropic drug naïve patients is of utmost importance (such as published by Prabakaran et al., 2007), the vast majority of samples available worldwide are derived from treated patients. As the samples used in the studies were derived from patients under distinct therapeutic regimens, the recurrent identification of the same targets might implicate certain genes and proteins in the pathobiology of the disease. Thus, we hope that our findings may not only reinforce the interest on the study of certain pathways, but also stimulate the study of the new potential markers uncovered here.

5. Author disclosure

The authors declare that they have no competing interests.

Conflict of interest

All authors declare that they have no conflicts of interest.

Contributors

This work is part of the PhD Thesis of DMS, who executed all the experiments and wrote the first manuscript draft. WFG and AS provided the brain samples, clinical data, and contributed to the manuscript writing and scientific discussions. GM, EHG, MNE and GHS provided support for mass spectrometry analysis as well as the data analysis and organization. SM and JCN provided the support for 2-DE analysis as well as the data analysis and organization. CWT provided guidance for the proteome analysis. EDN supervised this work and the manuscript writing. The study was conceived by DMS and EDN. All authors contributed to and have approved the final version of this manuscript.

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